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(54)【発明の名称】 ホスファチジルセリンの分画法

(57)【要約】

【課題】 天然あるいは人工的に調製されたリン脂質混  
合物からホスファチジルセリンを簡便に濃縮する分画法  
を得る。

【解決手段】 ホスファチジルセリンを含むリン脂質混  
合物をアルコール類に溶解した後、該溶解液中に金属塩  
を添加することによりホスファチジルセリンを不溶化せ  
しめ、該不溶部を分離するもの。

## 【特許請求の範囲】

【請求項1】 ホスファチジルセリンを含むリン脂質混合物をアルコール類に溶解した後、該溶解液中に金属塩を添加することによりホスファチジルセリンを不溶化せしめ、該不溶部を分離することを特徴とするホスファチジルセリンの分画法。

【請求項2】 前記金属塩として、リチウム塩、カリウム塩及びナトリウム塩から選ばれる1種又は2種以上を用いることを特徴とする請求項1に記載のホスファチジルセリンの分画法。

【請求項3】 前記金属塩として、塩化リチウム、塩化カリウム又は塩化ナトリウムを用いることを特徴とする請求項1又は2に記載のホスファチジルセリンの分画法。

【請求項4】 前記アルコール類として、エチルアルコールを用いることを特徴とする請求項1～3の何れかに記載のホスファチジルセリンの分画法。

## 【発明の詳細な説明】

## 【0001】

【発明の属する技術分野】 本発明は、リン脂質混合物からホスファチジルセリンを濃縮するための分画法に関するものである。

## 【0002】

【従来の技術】 ホスファチジルセリン（以下、「PS」と記載する。）は、痴呆症の予防や治療などを目的とした脳機能改善剤の他、免疫性疾患の治療剤や界面活性剤としての利用が期待されている。このPSは動物の脳や筋肉に含まれる他、化学合成法やホスホリバーゼDを使用したホスファチジル基転移反応により人工的に製造することも可能である。

【0003】 PSは主に医薬や食品、化粧品として使用されていることから、前記天然物や反応物等からPSを分画し、PS含量を高めることが重要である。ところが、動物の脳や筋肉に含まれるPS量は少なく、また化学合成法やホスファチジル基転移反応により製造した場合にも、PS含量の多い製品を安価に製造することは難しいことから、使用に際してはPSの濃縮（精製）が必要となる場合が多い。

【0004】 PSの濃縮（精製）法としては、従来、溶媒による分画やカラムクロマトグラフィーが用いられてきた。しかし、溶媒分画のみでは充分なPS純度を得ることは困難であり、一方、クロマトグラフィーのような煩雑な操作は、コスト面、作業性の面で問題があった。このため、PSを安価かつ簡便に濃縮する方法の開発が望まれている。

【0005】 特に、PSは他のリン脂質、すなわち、ホスファチジルコリン（PC）、ホスファチジルエタノールアミン（PE）、ホスファチジルイノシトール（PI）やホスファチジン酸（PA）等と分離することが困難であるため、これらを含むリン脂質混合物から、PS

を安価に濃縮する方法を確立することが望まれている。

## 【0006】

【発明が解決しようとする課題】 本発明者らは上記課題を解決するために銳意研究を重ねた結果、PSを含むリン脂質混合物をアルコール類に溶解し、さらに金属塩あるいはその溶液を添加することによりPSを沈殿せしめ、沈殿部に濃縮できることを見出した。

【0007】 本発明は、天然あるいは人工的に調製されたリン脂質混合物からPSを簡便に濃縮する分画法を得ることを目的とする。

## 【0008】

【課題を解決するための手段】 請求項1に記載された発明に係るPSの分画法は、PSを含むリン脂質混合物をアルコール類に溶解した後、該溶解液中に金属塩を添加することによりPSを不溶化せしめ、該不溶部を分離するものである。

【0009】 請求項2に記載された発明に係るPSの分画法は、請求項1に記載の金属塩として、リチウム塩、カリウム塩及びナトリウム塩から選ばれる1種又は2種以上を用いるものである。

【0010】 請求項3に記載された発明に係るPSの分画法は、請求項1又は2に記載の金属塩として、塩化リチウム、塩化カリウム又は塩化ナトリウムを用いるものである。

【0011】 請求項4に記載された発明に係るPSの分画法は、請求項1～3の何れかに記載のアルコール類として、エチルアルコールを用いるものである。

## 【0012】

【発明の実施の形態】 本発明においては、PSを含むリン脂質混合物をアルコール類に溶解した後、該溶解液中に金属塩を添加するという簡略な操作によって、PSを不溶化（沈殿、凝集等）させることにより、不溶物（沈殿部、凝集部等）を分離・濃縮する。これにより、天然あるいは人工的に調製されたリン脂質混合物からPSを簡便に濃縮することができる。

【0013】 本発明に用いられるPSを含むリン脂質混合物としては、天然物、天然物からの抽出物又は該抽出物を精製したもの、或いは合成リン脂質等PSを含む混合物であれば、いずれを用いてもよい。具体的には、大豆レシチン、菜種レシチン、卵黄レシチン、トウモロコシレシチン或いは綿実レシチンや、化学合成法やホスファチジル基転移反応により調製したリン脂質混合物、牛脳の溶媒抽出物等が挙げられる。中でも、ホスファチジル基転移反応により調製したPSを含むリン脂質混合物を用いれば、金属塩を添加した場合の濃縮効果が高く、原料の確保のしやすさやコスト面からも好ましい。

【0014】 また、本発明に用いられる金属塩としては、リチウム塩、ナトリウム塩、カリウム塩、カルシウム塩、マグネシウム塩等の金属塩、あるいはこれらを豊富に含む天然物、例えば、食塩、苦汁、かん水、ドロマ

イト、食用真珠層粉等いずれを用いても良いが、リチウム塩、ナトリウム塩またはカリウム塩を用いることが濃縮効率の点から好ましく、特に塩化リチウム、塩化ナトリウムまたは塩化カリウムが好ましい。これらの金属塩は、1種または2種以上を組み合わせて用いることができる。

【0015】これら金属塩の添加量は、PSを沈殿せ得る量であれば特に限定されないが、リン脂質1gあたり、0.15～1.0ミリモル、特に0.5～5ミリモルであることが、PSの回収率および沈殿中のPS含量が高い点から好ましい。

【0016】また、本発明に用いられるアルコール類としては、リン脂質混合物を溶解可能なアルコール類であればいずれも好適に用いられるが、中でもメチルアルコール、エチルアルコール、ブチルアルコール、プロピルアルコール、イソプロピルアルコール等の低級アルコール類が好ましい。また、これらの混合物を利用することもできるが、エチルアルコールは食品へ利用し易く、安全面での問題も少ないため、これを用いることが特に好ましい。

【0017】リン脂質混合物をアルコール類に溶解する際の濃度は特に限定されないが、この混合物が完全に溶解できる以上の量とすることが好ましく、アルコール類の重量に対し1～50%、特に2～20%とすることが、PS濃縮効率や操作性の点から好ましい。

【0018】本発明において、リン脂質混合物からのPSの分画は、例えば以下のようにして行うことができる。まず、ホスファチジル基転移反応法等により調製され、PC、PE又はPA等PS以外のリン脂質を成分中に含むリン脂質混合物を、エチルアルコール等のアルコール類に溶解する。この時、溶解温度等の溶解の条件は特に限定されず、混合物の成分の種類、それらの量等に合わせ好適な条件を選択し用いればよい。

【0019】こうして得られる溶液中では、PSやPC、PA等のリン脂質は溶媒層に抽出されるが、場合によっては一部の不溶性成分が生成する。このため、遠心分離、ろ過等の手段により溶媒から不溶性成分（沈殿物、凝集物等）を除いてから金属塩の添加を行う。不溶性成分中にも少量のPSが残存している場合には、前記アルコール類による抽出処理は、数度繰り返し行ってよい。

【0020】次いで、アルコール溶液に対して、金属塩を添加し、溶媒層に抽出されたPSを分画する。すなわち、溶媒層中のPS以外のリン脂質の大部分は、金属塩の添加によっても沈殿しないが、PSはその大部分が沈殿するため、これを回収することによりPSの濃縮を行うことができる。このとき、金属塩は粉末のまま加えても、水やアルコール等の溶媒に溶かしてから加えてもよい。その際の各種条件も特に限定されず、混合物の成分の種類、それらの量等に合わせ好適な条件を選択すれば

よい。具体的には10℃～30℃で30分以上保持してPSを不溶化させればよい。

【0021】金属塩の添加により不溶化されたPSは、遠心分離、ろ過、静置分離等の手段により、回収することができる。また、公知の精製手段、例えばカラムクロマトグラフィー等の手段により、更に精製することも可能である。本発明のPS濃縮物は他のリン脂質等の含量が顕著に低下しているため、このような精製手段も比較的簡便に行うことができる。

10 【0022】本発明のPS濃縮物は、医薬品、食品、化粧品等の形態で投与することができる。例えばリン脂質の生理効果を訴求する医薬品や栄養補助食品等の形態で用いる場合であれば、カプセル剤、顆粒剤、錠剤、散剤等の固形製剤、或いはシロップ剤等の液状製剤として経口投与することができる。また、経口投与剤でなくとも、注射剤、皮膚外用剤、直腸投与剤等非経口形態で投与することも可能である。

【0023】各製剤の製造時には、乳糖、澱粉、結晶セルロース、乳酸カルシウム、メタケイ酸アルミニン酸マグネシウム、無水ケイ酸等の賦形剤、白糖、ヒドロキシプロピルセルロース、ポリビニルピロリドン等の結合剤、カルボキシメチルセルロース、カルボキシメチルセルロースカルシウム等の崩壊剤、ステアリン酸マグネシウム、タルク、モノグリセリド、蔗糖脂肪酸エステル等の滑沢剤や、その他、医薬・食品として許容され得る成分を適宜使用すればよい。

【0024】また、同様の生理効果を期待して一般食品形態（「明らか食品」の形態）で用いる場合には、本発明の方法により得られたPS濃縮物をそのまま或いは適宜精製処理したものを油脂、錠菓、発酵乳、飴、調味料、ふりかけ等の飲食品に添加し、常法を用いて製造すればよい。

【0025】これら医薬品、食品等の形態での使用に際しては、本発明の方法により得られたPSが濃縮されたリン脂質組成物を適宜配合することができる。また、PSの生理効果を訴求する場合であれば、その効果を得られかづ過剰摂取等の問題が生じない程度の量、50mg～1000mg／日程度の摂取が見込まれる量を適宜配合しておけばよい。

40 【0026】更に、本発明のリン脂質は乳化剤として用いてもよく、その際には、医薬品、食品、化粧品等へ0.01～10%添加するのが好ましい。

【0027】

【実施例】以下に、実施例を挙げて本発明を説明するが、本発明はこれらに限定されるものではない。

【0028】実施例1

大豆レシチン（PC80：クロクラーン社製）10.0gと大豆油2.0gを100ccメジウム瓶に取り、ここに90.0g（100mL）の酢酸エチルを加えてスタートラーで攪拌しながら加温溶解した。Lーセリン1.

2 g と P L D - Y 1 ((株) ヤクルト本社製) 1, 50  
0 単位を秤り取り、0. 1 M リン酸ナトリウム緩衝液  
(pH 7. 0) 5. 8 mL を加えて溶解した。

【0029】 P C 8 0 溶液全量の入ったメジウム瓶を 5  
0 ℃ に保温しておき、ここに 50 ℃ に保温した (L-セ  
リン + P L D - Y 1) 溶液の全量を加えて反応を開始  
し、スターラーで緩やかに攪拌しながら 50 ℃ で 5 時間  
反応させた。30 分間氷冷してリン脂質を沈殿させて回  
収した後、熱湯中に 20 分放置して酵素を失活させた。

【0030】 回収したリン脂質層にエタノール 40 mL  
を加えて良く混合し、4 ℃ に一晩放置することにより沈  
殿を形成させ、上清を回収した。沈殿部にはさらにエタ  
ノール 12 mL を加えて良く混合後 30 分間放置し、遠  
心分離により上清を集め、先の上清と混合することにより  
大豆転移レシチン／エタノール溶液を得た。

【0031】 このようにして得た大豆転移レシチン／エ  
タノール溶液 2. 0 mL ( 固形分約 0. 33 g, リン脂  
質中の P S 含量 = 32. 5 % ) に 0. 1 M 酢酸ナトリウ  
ム／エタノール溶液を 1. 0 mL 加えて -20 ℃ で 1 時間  
放置し、生じた沈殿 (60 mg) を遠心操作により分  
離してエタノールで洗浄した (P p t N a - 1)。上清  
を -20 ℃ で数日間保存した結果、さらに沈殿 (7 m  
g) を生じたので遠心操作により上清 (S u p N a, 乾  
固重量 = 227 mg) と分離し、エタノールで洗浄した  
(P p t N a - 2)。

【0032】 各試料を乾固した後、希釈溶媒 (ヘキサ  
ン : ジエチルエーテル : イソプロパノール = 2 : 2 :  
1) に溶解し、薄層クロマトグラフィー ( 展開溶媒 : ク  
ロロホルム : メタノール : 酢酸 = 13 : 5 : 2 ) で展開  
してから、D i t t m e r - L e s t e r 試薬によりリ  
ン脂質を発色させ、ゲルパターン画像解析システムによ  
りリン脂質含量を定量した。結果を次の表 1 に示す。

【0033】 表 1 に示す通り、リン脂質中の P S 含量  
(モル%) は分画前は 32. 5 % であったのに対して、  
分画後の P p t N a では 81. 9 %, S u p N a では  
5. 6 % であることがわかり、P S が効率よく濃縮でき  
ることが確認された。

#### 【0034】

##### 【表 1】

各分画物のリン脂質組成 (モル%)

	P S	P A	P C	ツ' P C
大豆転移レシチン	32.4	15.4	44.3	8.1
P p t N a - 1	81.9	14.7	3.4	0.0
P p t N a - 2	57.2	20.0	20.5	2.3
S u p N a	5.6	6.8	75.6	11.9

#### 【0035】 実施例 2

実施例 1 で調製した大豆転移レシチン／エタノール溶液  
を減圧乾燥し、そのうちの 900 mg を 50 c c メジウ  
ム瓶に取り、クロロホルム 22. 5 mL とメタノール 1  
5 mL の混合液に溶解させた。こうして調製した大豆転  
移レシチン／クロメタ溶液を 4. 0 mL ずつ 6. 0 c c  
メジウム瓶に分注し、ここに 1 M 塩類溶液 (a. 塩化リ  
チウム、b. 塩化カリウム、c. 塩化ナトリウム、d.  
塩化マグネシウム、e. 塩化カルシウム、f. 塩化アン  
モニウム、g. 硫酸アンモニウム) を 0. 8 mL 加え  
た。瓶を振って数回混合した後、静置してクロロホルム  
層を回収し、その内の 1. 0 mL を秤量した試験管に取  
り空素下に乾燥した。

【0036】 こうして得られた乾燥物 ( 約 50 mg, リ  
ン脂質含量 = 約 25 mg) に対して 0. 10 mL のジエ  
チルエーテルを加えて溶解し、ここに 1. 0 mL のエタ  
ノールを徐々に加えて沈殿を形成させ、懸濁液全体を遠  
心分離して上清と沈殿とを分けた。

【0037】 エタノール抽出液は 2. 5 倍希釈液を 5  $\mu$   
L、沈殿は全体を 2. 5 mL のクロロホルムに溶解した  
もの 5  $\mu$  L を薄層板にアプライし、実施例 1 の条件で展  
開後、D i t t m e r - L e s t e r 試薬によりリン脂  
質を発色させゲルパターン画像解析システムによりリン  
脂質含量を定量した。結果を次の表 2 に示す。

【0038】 表 2 に示すように、使用した 8 種類の塩  
の中で塩化リチウムが最も成績が良く、1 回のエタノール  
沈殿により純度 80 % の P S 標品を得ることができた。  
さらに、塩化リチウムでは全 P A のうちの約 4 分の 3 が  
エタノール上清に分画されており、P A の除去効率が際  
立って良いことがわかった。

#### 【0039】

##### 【表 2】

## エタノール沈澱画分リン脂質中のPSおよびPA含量(%)

	PS	PA		PS	PA
塩化リチウム	79.8	8.1	塩化マグネシウム	52.8	18.7
塩化カリウム	75.7	14.0	塩化カルシウム	53.3	19.3
塩化ナトリウム	68.2	18.8	塩化アンモニウム	殆ど沈澱しない	
			硫酸アンモニウム	殆ど沈澱しない	

【0040】

\* \* 【表3】  
エタノール沈澱および上清画分中のリン脂質含量  
(画像解析装置により算出されたピーク面積として表示)

	PS			PA			PC		
	沈澱	上清	沈／上	沈澱	上清	沈／上	沈澱	上清	沈／上
塩化リチウム	9589	498	19.3	1038	3046	0.34	1098	11190	0.10
塩化カリウム	9911	1004	9.9	1959	1905	1.03	1184	11652	0.10
塩化ナトリウム	9487	914	10.3	2242	1682	1.33	1572	10409	0.15

【0041】なお、全体的な傾向としては陽イオンの原子番号が小さいほどPAの除去効率が良く、2価の陽イオンではPCも沈澱することがわかった。また、アンモニウム塩の場合にはPSを含む大部分のリン脂質が沈殿せず分離には適さなかった。沈殿の成績のよい塩化リチウム、塩化カリウム、塩化ナトリウムに関して、エタノール沈澱および上清画分中のリン脂質含量を表3に示す。

## 【0042】実施例3

実施例1で調製した大豆転移レシチン／エタノール溶液を減圧乾燥し、そのうちの900mgを50ccメジウム瓶に取り、クロロホルム22.5mLとメタノール15mLの混合液に溶解させた。こうして調製した大豆転移レシチン／クロロホルム－メタノール溶液に1M塩化リチウム溶液を8.0mL加え、瓶を振って数回混合した後、静置してクロロホルム層を回収し減圧乾固した。

【0043】得られた乾燥物に5.0mLのジエチルエーテルを加えて溶解し、ここに40mLのエタノールを徐々に加えて溶解し(20℃)、懸濁液全体を遠心分離して上清(S1)と沈澱(P1)とを分離した。P1に再び5.0mLのジエチルエーテルを加えて溶解し、こ

こに50mLのエタノールを徐々に加えて溶解し(20℃)、懸濁液全体を遠心分離して上清(S2)と沈澱(P2)とを分けた。

【0044】大豆転移レシチンは1.0mg/mL、P-2(湿潤状態)は2.0mg/mLのクロロホルムに溶解したものと5μL、S1はクロロホルムで2.5倍に希釈したものを5μL、そしてS2は原液10μLを薄層板にアプライし、実施例1の条件で展開後、Dittmer-Lester試薬によりリン脂質を発色させゲルパターン画像解析システムによりリン脂質含量を定量した。

【0045】エタノール沈澱物をもう一度エタノールで洗浄して得たP2画分中のPS含量は96.9%であった。CMセルロースを用いたイオン交換法における回収率は20%程度(参考例参照)であったが、本発明による分画法における回収率は約90%と高く、高純度品を用いた効率評価や作用機構の解明、あるいは医薬品開発に際しての有用な精製手段となり得る。

## 【0046】

【表4】

## 大豆転移PSリチウム塩のエタノール分画

	PS	PA	PC	Lyo PC	その他
大豆転移レシチン	36.6	12.2	40.4	7.6	4.2
P Z	96.9	1.7	0.0	0.0	1.4
S 1	6.8	13.7	66.7	9.3	3.4
S 2	41.0	18.2	34.7	2.8	3.3

## 【0047】実施例4

実施例1で調製した大豆転移レシチン／エタノール溶液を減圧乾燥し、そのうちの900mgを50ccメジウム瓶に取り、クロロホルム22.5mLとメタノール1.5mLの混合液に溶解させた。こうして調製した大豆転移レシチン／クロロホルム－メタノール溶液を4.0mLずつ6.0ccメジウム瓶に分注し、ここに種々のpHのクエン酸－クエン酸ナトリウム緩衝液(pH 2.8, 3.6, 4.1, 4.6, 5.0)に溶解した2M食塩水を0.8mL加えた。瓶を振って数回混合した後、静置してクロロホルム層を回収し、その内の2.5mLを秤量した試験管に取り窒素下に乾燥した。

【0048】乾燥物(約120mg)に対して0.2mLのジエチルエーテルを加えて溶解し、ここに2.0mLのエタノールを徐々に加えて溶解し、懸濁液全体を遠心分離して上清と沈殿とを分離した。エタノール抽出液は10倍希釈液を10μL、沈殿は全体を10.0mLのクロロホルムに溶解したものを5μL、薄層板にアブライし、実施例1の条件で展開後Dittmer-Lester試薬によりリン脂質を発色させ、ゲルパターン画像解析システムによりリン脂質含量を定量した。

【0049】pH 3.6以上の条件ではPSもPAも共に沈殿画分に回収され(PA/PS=0.23)、両者を分離することはできなかった。これに対してpH 2.8では沈殿に含まれるPA量が相対的に低く(PA/PS=0.15)、この条件でのエタノール処理を繰り返せばナトリウム塩の状態でPSを分画できる可能性が示された。

## 【0050】実施例5

PC含量40%の大豆レシチン200gにセリン水溶液 40

190g(セリン70g+水120g)とホスホリパーゼD(PLD-Y1、(株)ヤクルト本社製)の水溶液(24mg/mL)を10mL練り込んで55°Cで5時間反応させた結果、リン脂質中のPS含量が46.7%の反応生成物が得られた。

【0051】反応生成物5.0gにエチルアルコール20mLを加え45°Cで抽出後、残渣(沈殿)をさらにエチルアルコール5mLで2回抽出した。3回の抽出液を混合し、そのうちの5mLに25%食塩水0.20mLを加え、45°Cに加温後、室温に放置して沈殿を形成させた。その結果、上清中のPS含量は乾燥固体分中3.3%であったのに対して、沈殿物では62.1%であり、PSは沈殿部に効率よく濃縮されることがわかった。

## 【0052】実施例6

実施例5で調製した抽出液の混合物5mLに酢酸ナトリウム粉末50mgを加え45°Cに加温後、室温に放置して沈殿を形成させた。その結果、上清中のPS含量は乾燥固体分中3.5%であったのに対して、沈殿物では61.8%であり、食塩水を用いた場合と同じく、PSは沈殿部に効率よく濃縮されることがわかった。

## 【0053】実施例7

実施例5で調製した抽出液の混合物に対して25%食塩水を加えてPSの不溶物を形成させ、沈殿リン脂質中のPS含量と、沈殿部に回収されるPS量とを測定した。その結果として、食塩添加量と沈殿へのPS回収率及びリン脂質中のPS含量との関係を表5に示す。

## 【0054】

## 【表5】

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食塩添加量と沈殿へのPS回収率及びリン脂質中PS含量との関係

食塩添加量 (m moles/g リン脂質)	沈殿画分への PS回収率(%)	沈殿リン脂質中 PS含量(%)	上清リン脂質中 PS含量(%)
無添加	—	—	43.2
0.05	53.7	64.7	33.4
0.15	73.8	66.7	28.2
0.25	80.4	69.3	18.0
0.50	96.2	63.8	6.2
1.25	97.4	63.5	4.3
2.5	96.6	62.7	6.1
5	97.1	59.8	6.4
10	95.7	55.0	10.5
25	97.8	47.2	12.0
50	97.7	46.6	13.5

【0055】表5に示す通り、何れの条件でもPSは沈殿画分に濃縮されたが、特に抽出液混合物中のリン脂質1gあたりに加える食塩の量が10ミリモル以下の範囲において、沈殿リン脂質中のPS含量は55%以上であり、沈殿部にPSが効率よく濃縮されていた。一方、食塩添加量が0.05ミリモル以下の場合には、沈殿へのPSの回収率は60%以下であり、40%以上が上清部に存在していた。以上の結果から、何れの添加量でもPSを沈殿に濃縮することが可能であるが、アルコールに溶解したリン脂質1gに対する食塩の添加量が0.15~1.0ミリモルの範囲が特に実用に適した添加量と考えられた。

【0056】参考例 CM-セルロース・カラムクロマトグラフィーによる精製  
牛脳からのPSの精製例（新生化学実験講座4、脂質IIリン脂質、p. 127）に準じて実施例1で得た大豆転移レシチンからPSを精製することを試みた。

【0057】(1) Na+型CM-セルロースの調製：CM-52 (Whattmann社、膨潤型) 50gを0.5N水酸化ナトリウム500mL中に徐々に加えて約30分間静置した後に吸引濾過した。濾液が中性になるまで蒸留水で洗浄(500mL×2回)した後、0.5N塩酸500mLを流し蒸留水で濁液が中性になるまで再度洗浄(500mL×2回)した。このゲルを0.5N水酸化ナトリウム500mL中にかきまぜながら加え30分間静置したのち、蒸留水で中性になるまで洗浄(500mL×1回)し、さらにメタノール500mLで洗浄後、最終的にメタノール懸濁液として室温に保存した。

【0058】(2) クロマトグラフィー：(1)で調整したCM-52を内径30mmのカラムに充填(ベッド体積70mL)し、クロロホルムを500mL流してコンディショニングした後、実施例1の大転移レシチン1.0gを10mLのクロロホルムに溶解して沈殿を除いた溶液をカラムにアプライした後、クロロホルムで

溶出させ、200mLのクロロホルム溶出画分(Fr. 1)を得た。

【0059】更に、クロロホルム-メタノール(85:15, v/v)400mL(Fr. 2+3)、クロロホルム-メタノール(75:25, v/v)400mL(Fr. 4+5)、クロロホルム-メタノール(65:35, v/v)400mL(Fr. 6+7)、クロロホルム-メタノール(50:50, v/v)400mL(Fr. 8+9)を流して200mLずつを分画し、シリカゲル薄層クロマトグラフィーによりリン脂質を分析した。

【0060】その結果、クロロホルム溶出画分(Fr. 1)にはほとんどリン脂質が検出されなかったが、クロロホルム-メタノール(85:15, v/v)画分の前半(Fr. 2)には白濁状態でアプライしたエタノール沈殿物とほぼ同じ組成の物が0.29g(乾燥重量)溶出された。クロロホルム-メタノール(75:25, v/v)画分(Fr. 4+5)にはPCは含まれなかつたがPSとPAの両方が含まれており、特にPAが多く溶出されていた。そして、クロロホルム-メタノール(65:35, v/v)画分の前半(Fr. 6)にはまだ8.0%のPAが含まれていたが、後半(Fr. 7)以後にはPAが含まれておらず、PS以外には原点にわずかの発色が見られるのみで純度は97.7%であった。なお、クロロホルム-メタノール(50:50, v/v)画分の前半(Fr. 8)には相当量のPSが含まれていたが、後半(Fr. 9)にはほとんど溶出物がなかった。

【0061】以上の結果から、大豆転移レシチンからも牛脳分画物とほぼ同様の条件の陽イオン交換クロマトによりPSを精製できることが明らかになったが、回収率は極めて低く(Fr. 7以後のみを回収した場合、20%程度)、大量の精製PSを得るには適さない方法であることがわかった。

【0062】

【発明の効果】本発明は以上説明した通り、天然あるいは  
人工的に調製されたリン脂質混合物からP Sを簡便に＊  
＊濃縮する分画法を得ることができるという効果がある。

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フロントページの続き

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**CLAIMS DETAILED DESCRIPTION TECHNICAL FIELD PRIOR ART EFFECT OF THE  
INVENTION TECHNICAL PROBLEM MEANS EXAMPLE**

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[Translation done.]

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**CLAIMS**

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[Claim(s)]

[Claim 1]A fractionation method of phosphatidylserine making phosphatidylserine insolubilize and separating this insoluble part by adding metal salt in this solution after dissolving a phospholipid mixture containing phosphatidylserine in alcohols.

[Claim 2]A fractionation method of the phosphatidylserine according to claim 1 using one sort chosen from lithium salt, potassium salt, and sodium salt, or two sorts or more as said metal salt.

[Claim 3]A fractionation method of the phosphatidylserine according to claim 1 or 2 characterized by using a lithium chloride, potassium chloride, or sodium chloride as said metal salt.

[Claim 4]A fractionation method of the phosphatidylserine according to any one of

claims 1 to 3 characterized by using ethyl alcohol as said alcohols.

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[Translation done.]

## DETAILED DESCRIPTION

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[Detailed Description of the Invention]

[0001]

[Field of the Invention] This invention relates to the fractionation method for condensing phosphatidylserine from a phospholipid mixture.

[0002]

[Description of the Prior Art] The use as the treating agent and surface-active agent of an immune disease besides the cerebral function reforming agent [ phosphatidylserine / (it is hereafter indicated as "PS") ] aiming at prevention, a therapy, etc. of dementia is expected. This PS is contained in the brain and muscles of an animal, and also it can be artificially manufactured by the transphosphatidylation which uses a chemosynthesis method and phospholipase D.

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[0003] Since PS is mainly used as medicine, foodstuffs, and cosmetics, it is important for it to carry out fractionation of the PS from said natural product, a reactant, etc., and to raise PS content. However, since it is difficult to manufacture a product with many PS contents cheaply, when using it, concentration (refining) of PS is needed [ there are few amounts of PS contained in the brain and muscles of an animal, and ], also when it manufactures by a chemosynthesis method or transphosphatidylation in many cases.

[0004] As a method of condensing PS (refining), the fractionation and column chromatography by a solvent have been used conventionally. However, it is difficult to obtain PS purity sufficient by just solvent fractionation, and, on the other hand, complicated operation like chromatography had a problem in respect of a cost aspect and workability. For this reason, development of the method of condensing PS cheaply and simple is desired.

[0005] Since especially PS is difficult to separate from other phospholipid (PC), i.e., phosphatidylcholine, phosphatidylethanolamine (PE), phosphatidylinositol (PI),

phosphatidic acid (PA), etc., To establish the method of condensing PS cheaply from the phospholipid mixture containing these is desired.

[0006]

[Problem(s) to be Solved by the Invention] This invention persons dissolved the phospholipid mixture containing PS in alcohols, as a result of repeating research wholeheartedly, in order to solve an aforementioned problem, and they made PS precipitate by adding metal salt or its solution further, and it found out that it could condense to a precipitating part.

[0007] An object of this invention is to acquire the fractionation method which condenses PS simple from the phospholipid mixture prepared nature or artificially.

[0008]

[Means for Solving the Problem] After a fractionation method of PS concerning an invention indicated to claim 1 dissolves a phospholipid mixture containing PS in alcohols, by adding metal salt in this solution, it makes PS insolubilize and separates this insoluble part.

[0009] One sort chosen from lithium salt, potassium salt, and sodium salt or two sorts or more are used for a fractionation method of PS concerning an invention indicated to claim 2 as the metal salt according to claim 1.

[0010] A lithium chloride, potassium chloride, or sodium chloride is used for a fractionation method of PS concerning an invention indicated to claim 3 as the metal salt according to claim 1 or 2.

[0011] Ethyl alcohol is used for a fractionation method of PS concerning an invention indicated to claim 4 as the alcohols according to any one of claims 1 to 3.

[0012]

[Embodiment of the Invention] In this invention, after dissolving the phospholipid mixture containing PS in alcohols, insoluble matters (a precipitating part, a flocculation part, etc.) are separated and condensed by making PS insolubilize (precipitate, condensation, etc.) by simple operation of adding metal salt in this solution. PS can be condensed simple from the phospholipid mixture prepared nature or artificially by this.

[0013] As a phospholipid mixture containing PS used for this invention, as long as it is a mixture containing PS, such as a thing which refined the extract or this extract from a natural product and a natural product, or synthetic phospholipid, any may be used. Specifically, a phospholipid mixture, a solvent extraction thing of a bovine brain, etc.

which were prepared by a soybean lecithin, rapeseed lecithin, yolk lecithin, corn lecithin or cottonseed lecithin, a chemosynthesis method, or transphosphatidylation are mentioned. Especially, if the phospholipid mixture containing PS prepared by transphosphatidylation is used, the degree of enrichment at the time of adding metal salt is high, and preferred also from the ease of carrying out and cost aspect of reservation of a raw material.

[0014]As metal salt used for this invention, lithium salt, sodium salt, Metal salt, such as potassium salt, calcium salt, and magnesium salt, or the natural product which contains these abundantly, For example, although salt, bittern, saline water, dolomite, edible mother-of-pearl powder, etc. may use any, it is preferred from a point of condensation efficiency to use lithium salt, sodium salt, or potassium salt, and especially a lithium chloride, sodium chloride, or potassium chloride is preferred. These metal salt can be used combining one sort or two sorts or more.

[0015]Especially if the addition of these metal salt is the quantity which may settle PS, it will not be limited, but it is [ the recovery rate of PS and its PS content under precipitate ] preferred from a high point per [ phospholipid 1g ] and that it is 0.5 to 5 millimol especially 0.15 to 10 millimol.

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[0016]If it is alcohols which can dissolve a phospholipid mixture as alcohols used for this invention, all will be used suitably, but. Lower alcohol, such as methyl alcohol, ethyl alcohol, butyl alcohol, propyl alcohol, and isopropyl alcohol, is especially preferred. Although these mixtures can also be used, it is easy to use it for foodstuffs, and since ethyl alcohol also has few problems in a safety aspect, especially the thing for which this is used is preferred [ ethyl alcohol ].

[0017]Although the concentration in particular at the time of dissolving a phospholipid mixture in alcohols is not limited, it is preferred to consider it as the above quantity that this mixture can dissolve thoroughly, and it is preferred from PS condensation efficiency or a point of operativity to consider it as 2 to 20% especially 1 to 50% to the weight of alcohols.

[0018]In this invention, fractionation of PS from a phospholipid mixture can be performed as follows, for example. First, it is prepared by a transphosphatidylation method etc. and the phospholipid mixture which contains phospholipid other than PS, such as PC, PE, or PA, in an ingredient is dissolved in alcohols, such as ethyl alcohol. At this time, the conditions in particular of the dissolution of a melting temperature etc.

are not limited, but are doubled with the kinds of ingredient of a mixture, those quantity, etc., and should just choose and use suitable conditions.

[0019]In this way, in the solution obtained, although phospholipid, such as PS, PC, PA, is extracted by the solvent layer, depending on the case, some insoluble components generate it. For this reason, metal salt is added after removing insoluble components (a sediment, an aggregate, etc.) from a solvent by centrifugal separation, filtration, or other means. When a little PS remains also in an insoluble component, the extracting processing by said alcohols is good in an abundance repetition line.

[0020]Subsequently, to an alcohol solution, metal salt is added and fractionation of the PS extracted by the solvent layer is carried out. That is, although the great portion of phospholipid other than PS in a solvent layer does not precipitate by addition of metal salt, either, since the most precipitates, PS can condense PS by collecting these. Even if it adds metal salt with powder at this time, it may add, after melting in solvents, such as water and alcohol. What is necessary is not to limit the various conditions in particular in that case, either, but to double with the kinds of ingredient of a mixture, those quantity, etc., and just to choose suitable conditions. What is necessary is to specifically hold 30 minutes or more at 10 \*\* - 30 \*\*, and just to make PS insolubilize.

[0021]PS insolubilized by addition of metal salt is recoverable by centrifugal separation, filtration, settlement separation, or other means. It is also possible to refine further by the publicly known refining means, for example, column chromatography etc., or other means. Since the content of other phospholipid is falling notably, PS concentrate of this invention can be performed comparatively simple [ such a refining means ].

[0022]PS concentrate of this invention can be prescribed for the patient with the gestalt of drugs, foodstuffs, cosmetics, etc. For example, if it is a case where it uses with the gestalt of the drugs which solicit the physiology effect of phospholipid, a supplement, etc., it can administer orally as liquid preparations, such as solid preparations, such as a capsule, a granule, a tablet, and powder medicine, or syrups. Even if it is not an orally administered drug, it is also possible to prescribe a medicine for the patient with parenteral gestalten, such as injections, skin external preparations, and a rectum administration agent.

[0023]At the time of manufacture of each pharmaceutical preparation, milk sugar, starch, crystalline cellulose, calcium lactate, Excipients, such as magnesium aluminometasilicate and a silicic acid anhydride, white soft sugar, Binding materials,

such as hydroxypropylcellulose and a polyvinyl pyrrolidone, What is necessary is just to use suitably lubricant, such as disintegrator, such as carboxymethyl cellulose and carboxymethyl-cellulose calcium, magnesium stearate, talc, monoglyceride, and a sucrose fatty acid ester, and the other ingredients which may be permitted as medicine and foodstuffs.

[0024]What is necessary is to add what carried out purification treatment of the PS concentrate obtained by the method of this invention as it is or suitably in eating-and-drinking articles, such as fats and oils, a hard candy, fermented milk, a candy, a seasoning, and fish flour, and just to manufacture using a conventional method, in expecting the same physiology effect and using with a common foodstuffs gestalt (gestalt of "clear foodstuffs").

[0025]On the occasion of use with the gestalt of these drugs, foodstuffs, etc., the phospholipid composition in which PS obtained by the method of this invention was condensed can be blended suitably. what is necessary is just to blend suitably the quantity which is a grade which can acquire the effect and problems, such as superfluous ingestion, do not produce, and the quantity the ingestion which is 50 mg – about 1000mg/day is expected to be, if it is a case where the physiology effect of PS is solicited

[0026]As for phospholipid of this invention, it is preferred to use as an emulsifier and to add 0.01 to 10% to drugs, foodstuffs, cosmetics, etc. in that case.

[0027]

[Example]Although an example is given to below and this invention is explained to it, this invention is not limited to these.

[0028]while taking 10.0 g of example 1 soybean lecithins (PC80: made by clo KURAN), and the soybean oil 2.0g into a 100-cc medium bottle, adding 90.0 g (100mL) ethyl acetate here and agitating with a stirrer — warming — it dissolved. \*\*\*\* picking and 0.1M sodium phosphate buffer solution (pH 7.0) 5.8mL was added, and L-serine 1.2g and PLD-Y1 (Made by Yakult Honsha) 1,500 unit were dissolved.

[0029]The medium bottle containing PC80 solution whole quantity is kept warm at 50 \*\*, the whole quantity of the solution (L-serine+PLD-Y1) which kept it warm at 50 \*\* is added here, and a reaction is started, and it was made to react at 50 \*\* for 5 hours, stirring gently with a stirrer. After ice-cooling for 30 minutes, settling phospholipid and collecting, in boiling water, it was neglected for 20 minutes and the enzyme was

deactivated.

[0030]Ethanol 40mL could be added to the collected phospholipid layer, and it mixed, and by neglecting it at 4 \*\* overnight, precipitate was made to form and supernatant liquid was collected. Ethanol 12mL could be further added to the precipitating part, it was neglected for 30 minutes after mixing, supernatant liquid was collected by centrifugal separation, and soybean transition lecithin / ethanol solution was obtained by mixing with previous supernatant liquid.

[0031]Thus, add 0.1M sodium acetate / ethanol solution to the obtained soybean transition lecithin / ethanol solution 2.0mL (about 0.33 g of solid content, PS content in phospholipid = 32.5%) 1.0 mL, and it is neglected at -20 \*\* for 1 hour, Centrifugal operation separated the formed precipitate (60 mg), and it washed by ethanol (PptNa-1). As a result of saving supernatant liquid for several days at -20 \*\*, since the precipitate (7 mg) was produced further, centrifugal operation separated with supernatant liquid (SupNa, hardening-by-drying weight = 227 mg), and it washed by ethanol (PptNa-2).

[0032]After hardening each sample by drying, it dissolves in a diluent solvent (hexane: diethylether : isopropanol =2:2:1), After developing with thin-layer chromatography (chloroform: developing solvent : methanol : acetic acid =13:5:2), under [ a fixed quantity / analysis system / gel pattern image / content / phospholipid / it makes phospholipid color with a Dittmer-Lester reagent and ]. A result is shown in the next table 1.

[0033]It was checked that, as for before fractionation, it turns out to having been 32.5% that it is [ which it is 81.9% in PptNa after fractionation ] 5.6% in SupNa, and PS can condense PS content (mol %) in phospholipid efficiently as shown in Table 1.

[0034]

[Table 1]

[0035]Reduced pressure drying of the soybean transition lecithin / the ethanol solution prepared in example 2 Example 1 was carried out, 900 mg of them was taken into the 50-cc medium bottle, and it was made to dissolve in the mixed liquor of chloroform 22.5mL and methanol 15mL. In this way, the prepared soybean transition lecithin / clo meta-solution are poured distributively into every 4.0 mL 6.0-cc medium bottle, 1M saline (a. a lithium chloride, b. potassium chloride, c. sodium chloride, d. magnesium chloride, e. calcium chloride, f. ammonium chloride, g. ammonium sulfate) was added here 0.8 mL. After shaking the bottle and mixing several times, it settled, chloroform layers were collected, and it took in the test tube which carried out weighing of the 1.0mL of them, and dried under nitrogen.

[0036]In this way, to the obtained dry matter (about 50 mg, a phospholipid content = about 25 mg), added 0.10-ml diethylether and dissolved, and added the ethanol of 1.0mL here gradually, precipitation was made to form, the whole suspension was centrifuged, and supernatant liquid and precipitation were divided.

[0037]An ethanol extract applies 5micro of things L by which 5microL and precipitation dissolved the whole for the diluent in chloroform of 2.5mL 2.5 times to a laminated plate, Under [ a fixed quantity / analysis system / gel pattern image / content / phospholipid / it makes phospholipid color after deployment and with a Dittmer-Lester reagent on condition of Example 1 and ]. A result is shown in the next table 2.

[0038]As shown in Table 2, the lithium chloride was the highest-achieving of eight kinds of the used salts, and PS preparation of 80% of purity was able to be obtained by the ethanol precipitation which is 1 time. It turned out that fractionation of about 3/4 of all the PA is carried out to ethanol supernatant liquid, and the removal efficiency of PA may be conspicuous for a lithium chloride.

[0039]

[Table 2]

[0040]

[Table 3]

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[0041]The removal efficiency of PA was so good that the atomic number of the positive ion was small as an overall trend, and it turned out in the divalent positive ion that PC also precipitates. Phospholipid of most which contains PS in the case of ammonium salt did not precipitate, and it was not suitable for separation. The phospholipid content in ethanol precipitation and a supernatant liquid fraction is shown in Table 3 about the high-achieving lithium chloride of precipitate, potassium chloride, and sodium chloride.

[0042]Reduced pressure drying of the soybean transition lecithin / the ethanol solution prepared in example 3 Example 1 was carried out, 900 mg of them was taken into the 50-cc medium bottle, and it was made to dissolve in the mixed liquor of chloroform 22.5mL and methanol 15mL. In this way, 1M lithium-chloride solution was added to the prepared soybean transition lecithin / chloroform methanol solution 8.0 mL, after

shaking the bottle and mixing several times, it settled, and chloroform layers were collected and decompression hardening by drying was carried out.

[0043]Diethylether of 5.0mL was added to the obtained dry matter, and it dissolved in it, and the ethanol of 40mL was added here gradually, it dissolved (20 \*\*), the whole suspension was centrifuged, and supernatant liquid (S1) and precipitation (P1) were separated. Diethylether of 5.0mL was again added to P1, and it dissolved in it, and the ethanol of 50mL was added here gradually, it dissolved (20 \*\*), the whole suspension was centrifuged, and supernatant liquid (S2) and precipitation (P2) were divided.

[0044]That to which 5microL and S1 diluted with chloroform what soybean transition lecithin dissolved in 10 mg/mL, and dissolved P2 (damp or wet condition) in chloroform of 20 mg/mL 2.5 times 5microL, And apply undiluted solution 10muL to a laminated plate, phospholipid is made to color after deployment and with a Dittmer-Lester reagent on condition of Example 1, and S2 is the bottom about a fixed quantity of phospholipid contents by a gel pattern image analysis system.

[0045]PS content in P2 fraction which washed and obtained the ethanol precipitation thing by ethanol once again was 96.9%. Although the recovery rate in the ionic exchange method using CM cellulose was about (refer to reference example) 20%, the recovery rate in the fractionation method by this invention can serve as a useful refining means for about 90%, the effect evaluation it is high and using the high grade article, a break through of the mechanism of action, or drugs development.

[0046]

[Table 4]

[0047]Reduced pressure drying of the soybean transition lecithin / the ethanol solution prepared in example 4 Example 1 was carried out, 900 mg of them was taken into the

50-cc medium bottle, and it was made to dissolve in the mixed liquor of chloroform 22.5mL and methanol 15mL. In this way, the prepared soybean transition lecithin / chloroform methanol solution were poured distributively into every 4.0 mL 6.0-cc medium bottle, and 2M salt solution which dissolved in the citrate-sodium-acid-citrate buffer solution (pH 2.8, 3.6, 4.1, 4.6, 5.0) of various pH was added here 0.8 mL. After shaking the bottle and mixing several times, it settled, chloroform layers were collected, and it took in the test tube which carried out weighing of the 2.5mL of them, and dried under nitrogen.

[0048]To the dry matter (about 120 mg), diethylether of 0.2mL was added and it dissolved, and the ethanol of 2.0mL was added here gradually, it dissolved, the whole suspension was centrifuged, and supernatant liquid and precipitation were separated. An ethanol extract that by which 10microL and precipitation dissolved the whole for the diluent in chloroform of 10.0mL 10 times 5microL, Under [ a fixed quantity / analysis system / gel pattern image / content / phospholipid / apply to a laminated plate, and it makes phospholipid color with an after-deployment Dittmer-Lester reagent on condition of Example 1 and ].

[0049]On pH 3.6 or more conditions, both PS and PA were collected by the precipitation fraction (PA/PS=0.23), and were not able to separate both. On the other hand, a possibility that it could carry out fractionation of the PS in the state of sodium salt low (PA/PS=0.15) relatively if the amount of PA contained in precipitation repeats the ethanol treatment in this condition was shown by pH 2.8.

[0050]The result of having scoured the serine solution 190g (serine 70g+ water 120g) and the solution (24 mg/mL) of phospholipase D (PLD-Y1, Yakult Honsha Make) to the soybean lecithin 200g of 40% of the example 5PC content 10 mL, and having made them reacting to it at 55 \*\* for 5 hours, The resultant whose PS content in phospholipid is 46.7% was acquired.

[0051]Ethyl alcohol 20mL was added to the resultant 5.0g, and ethyl alcohol 5mL extracted residue (precipitate) twice further after extraction at 45 \*\*. 3 times of extracts are mixed, salt solution 0.20mL is added to 5mL of them 25%, it was neglected to the room temperature and precipitate was made to form after warming at 45 \*\*. As a result, it turned out that it is 62.1% in a sediment and PS is efficiently condensed by the precipitating part to PS content in supernatant liquid having been 3.3% among the dry solid.

[0052]50 mg of sodium acetate powder is added to mixture 5mL of the extract prepared in example 6 Example 5, it was neglected to the room temperature and precipitate was made to form after warming at 45 \*\*. As a result, it turned out that PS is efficiently condensed by the precipitating part as well as the case where are 61.8% in a sediment and a salt solution is used to PS content in supernatant liquid having been 3.5% among the dry solid.

[0053]Added the salt solution 25% to the mixture of an extract prepared in example 7 Example 5, the insoluble matter of PS was made to form, and PS content in precipitate phospholipid and the amount of PS collected by the precipitating part were measured. As the result, the relation between a salt addition, PS recovery rate to precipitate, and PS content in phospholipid is shown in Table 5.

[0054]

[Table 5]

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[0055]PS was condensed by the precipitate fraction also on condition of any as shown in Table 5, but the quantity of the salt especially added to per [ phospholipid 1g in an extract mixture ] of PS content in precipitate phospholipid is not less than 55% in the range of 10 or less millimols, and PS was efficiently condensed by the precipitating part. On the other hand, when a salt addition was 0.05 or less millimols, the recovery rate of PS to precipitate is 60% or less, and not less than 40% existed in the supernatant liquid part. Although it was possible to have condensed PS from the above result to

precipitate with any addition, the addition of the salt to the phospholipid 1g which dissolved in alcohol was considered to be the addition to which especially the range of 0.15 to 10 millimol was suitable for practical use.

[0056]Reference example It tried to refine PS from the soybean transition lecithin obtained in Example 1 according to the example of refining of PS from the refining bovine brains by CM cellulose column chromatography (the new chemical experiment lecture 4, lipid II phospholipid, p.127).

[0057](1) Preparation of Na<sup>+</sup> type CM cellulose : suction \*\*\*\* was carried out, after adding CM-52 (Whatmann, swollen type) 50g gradually and settling it for about 30 minutes into 0.5N sodium hydroxide 500mL. It washed again after distilled water washed until \*\*\*\* became neutrality (500mLx2 time) until it passed 0.5N chloride 500mL and \*\*\*\* became neutrality with distilled water (500mLx2 time). After settling for 30 minutes in addition, stirring this gel in 0.5N sodium hydroxide 500mL, it washed until it became neutrality with distilled water (500mLx1 time), and saved as methanol suspension eventually after washing by methanol 500mL further at the room temperature.

[0058](2) A column 30 mm in inside diameter is filled up with CM-52 adjusted by chromatography: (1) (bed volume 70mL), After having dissolved the soybean transition lecithin 1.0g of Example 1 in chloroform of 10mL after 500-mL-pouring chloroform and carrying out a conditioning, and applying the solution except precipitation to a column, you made it eluted under chloroform and the chloroform eluate fraction (Fr.1) of 200mL was obtained.

[0059]Chloroform methanol (85:15, v/v) 400mL (Fr.2+3), Chloroform methanol (75:25, v/v) 400mL (Fr.4+5), Chloroform methanol (65:35, v/v) 400mL (Fr.6+7) and chloroform methanol (50:50, v/v) 400mL (Fr.8+9) were passed, fractionation of every 200 mL was carried out, and silica gel thin layer chromatography analyzed phospholipid.

[0060]As a result, although phospholipid was hardly detected by the chloroform eluate fraction (Fr.1), 0.29g (dry weight) elution of the thing of the almost same presentation as the ethanol precipitation thing applied by the cloudy state was carried out in the first half (Fr.2) of a chloroform methanol (85:15, v/v) fraction. Although PC was not contained in the chloroform methanol (75:25, v/v) fraction (Fr.4+5), both PS and PA are contained, and it was mostly eluted especially in PA. And purity was [ that PA is not contained on and after the second half (Fr.7), but coloring slight at the starting point in

addition to PS is only seen, and ] 97.7% although 8.0% of PA was still contained in the first half (Fr.6) of a chloroform methanol (65:35, v/v) fraction. Although a considerable amount of PS was contained in the first half (Fr.8) of a chloroform methanol (50:50, v/v) fraction, there was almost no effluent in the second half (Fr.9).

[0061]Although it became clear from the above result that PS can be refined also from soybean transition lecithin by the cation-exchange chromatography of the almost same conditions as a bovine brain fraction, It turned out that it is the method of not being suitable for a recovery rate obtaining a lot of refining PS very low (it is about 20% when Fr.7 or subsequent ones are collected).

[0062]

[Effect of the Invention]This invention is effective in the ability to acquire the fractionation method which condenses PS simple from the phospholipid mixture prepared nature or artificially as it was explained above.

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[Translation done.]

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## TECHNICAL FIELD

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[Field of the Invention]This invention relates to the fractionation method for condensing phosphatidylserine from a phospholipid mixture.

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[Translation done.]

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## PRIOR ART

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[Description of the Prior Art]The use as the treating agent and surface-active agent of an immune disease besides the cerebral function reforming agent [ phosphatidylserine / (it is hereafter indicated as "PS".) ] aiming at prevention, a therapy, etc. of dementia is expected. This PS is contained in the brain and muscles of an animal, and also it can

be artificially manufactured by the transphosphatidylation which uses a chemosynthesis method and phospholipase D.

[0003] Since PS is mainly used as medicine, foodstuffs, and cosmetics, it is important for it to carry out fractionation of the PS from said natural product, a reactant, etc., and to raise PS content. However, since it is difficult to manufacture a product with many PS contents cheaply, when using it, concentration (refining) of PS is needed [there are few amounts of PS contained in the brain and muscles of an animal, and], also when it manufactures by a chemosynthesis method or transphosphatidylation in many cases.

[0004] As a method of condensing PS (refining), the fractionation and column chromatography by a solvent have been used conventionally. However, it is difficult to obtain PS purity sufficient by just solvent fractionation, and, on the other hand, complicated operation like chromatography had a problem in respect of a cost aspect and workability. For this reason, development of the method of condensing PS cheaply and simple is desired.

[0005] Since especially PS is difficult to separate from other phospholipid (PC), i.e., phosphatidylcholine, phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), etc., To establish the method of condensing PS cheaply from the phospholipid mixture containing these is desired.

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[Translation done.]

## EFFECT OF THE INVENTION

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[Effect of the Invention] This invention is effective in the ability to acquire the fractionation method which condenses PS simple from the phospholipid mixture prepared nature or artificially as it was explained above.

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[Translation done.]

## TECHNICAL PROBLEM

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[Problem(s) to be Solved by the Invention] This invention persons dissolved the phospholipid mixture containing PS in alcohols, as a result of repeating research wholeheartedly, in order to solve an aforementioned problem, and they made PS precipitate by adding metal salt or its solution further, and it found out that it could condense to a precipitating part.

[0007] An object of this invention is to acquire the fractionation method which condenses PS simple from the phospholipid mixture prepared nature or artificially.

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[Translation done.]

## MEANS

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[Means for Solving the Problem] After a fractionation method of PS concerning an invention indicated to claim 1 dissolves a phospholipid mixture containing PS in alcohols, by adding metal salt in this solution, it makes PS insolubilize and separates this insoluble part.

[0009] One sort chosen from lithium salt, potassium salt, and sodium salt or two sorts or more are used for a fractionation method of PS concerning an invention indicated to claim 2 as the metal salt according to claim 1.

[0010] A lithium chloride, potassium chloride, or sodium chloride is used for a fractionation method of PS concerning an invention indicated to claim 3 as the metal salt according to claim 1 or 2.

[0011] Ethyl alcohol is used for a fractionation method of PS concerning an invention indicated to claim 4 as the alcohols according to any one of claims 1 to 3.

[0012]

[Embodiment of the Invention] In this invention, after dissolving the phospholipid mixture containing PS in alcohols, insoluble matters (a precipitating part, a flocculation

part, etc.) are separated and condensed by making PS insolubilize (precipitate, condensation, etc.) by simple operation of adding metal salt in this solution. PS can be condensed simple from the phospholipid mixture prepared nature or artificially by this. [0013]As a phospholipid mixture containing PS used for this invention, as long as it is a mixture containing PS, such as a thing which refined the extract or this extract from a natural product and a natural product, or synthetic phospholipid, any may be used. Specifically, a phospholipid mixture, a solvent extraction thing of a bovine brain, etc. which were prepared by a soybean lecithin, rapeseed lecithin, yolk lecithin, corn lecithin or cottonseed lecithin, a chemosynthesis method, or transphosphatidylation are mentioned. Especially, if the phospholipid mixture containing PS prepared by transphosphatidylation is used, the degree of enrichment at the time of adding metal salt is high, and preferred also from the ease of carrying out and cost aspect of reservation of a raw material.

[0014]As metal salt used for this invention, lithium salt, sodium salt, Metal salt, such as potassium salt, calcium salt, and magnesium salt, or the natural product which contains these abundantly, For example, although salt, bittern, saline water, dolomite, edible mother-of-pearl powder, etc. may use any, it is preferred from a point of condensation efficiency to use lithium salt, sodium salt, or potassium salt, and especially a lithium chloride, sodium chloride, or potassium chloride is preferred. These metal salt can be used combining one sort or two sorts or more.

[0015]Especially if the addition of these metal salt is the quantity which may settle PS, it will not be limited, but it is [ the recovery rate of PS and its PS content under precipitate ] preferred from a high point per [ phospholipid 1g ] and that it is 0.5 to 5 millimol especially 0.15 to 10 millimol.

[0016]If it is alcohols which can dissolve a phospholipid mixture as alcohols used for this invention, all will be used suitably, but. Lower alcohol, such as methyl alcohol, ethyl alcohol, butyl alcohol, propyl alcohol, and isopropyl alcohol, is especially preferred. Although these mixtures can also be used, it is easy to use it for foodstuffs, and since ethyl alcohol also has few problems in a safety aspect, especially the thing for which this is used is preferred [ ethyl alcohol ].

[0017]Although the concentration in particular at the time of dissolving a phospholipid mixture in alcohols is not limited, it is preferred to consider it as the above quantity that this mixture can dissolve thoroughly, and it is preferred from PS condensation

efficiency or a point of operativity to consider it as 2 to 20% especially 1 to 50% to the weight of alcohols.

[0018]In this invention, fractionation of PS from a phospholipid mixture can be performed as follows, for example. First, it is prepared by a transphosphatidylation method etc. and the phospholipid mixture which contains phospholipid other than PS, such as PC, PE, or PA, in an ingredient is dissolved in alcohols, such as ethyl alcohol. At this time, the conditions in particular of the dissolution of a melting temperature etc. are not limited, but are doubled with the kinds of ingredient of a mixture, those quantity, etc., and should just choose and use suitable conditions.

[0019]In this way, in the solution obtained, although phospholipid, such as PS, PC, PA, is extracted by the solvent layer, depending on the case, some insoluble components generate it. For this reason, metal salt is added after removing insoluble components (a sediment, an aggregate, etc.) from a solvent by centrifugal separation, filtration, or other means. When a little PS remains also in an insoluble component, the extracting processing by said alcohols is good in an abundance repetition line.

[0020]Subsequently, to an alcohol solution, metal salt is added and fractionation of the PS extracted by the solvent layer is carried out. That is, although the great portion of phospholipid other than PS in a solvent layer does not precipitate by addition of metal salt, either, since the most precipitates, PS can condense PS by collecting these. Even if it adds metal salt with powder at this time, it may add, after melting in solvents, such as water and alcohol. What is necessary is not to limit the various conditions in particular in that case, either, but to double with the kinds of ingredient of a mixture, those quantity, etc., and just to choose suitable conditions. What is necessary is to specifically hold 30 minutes or more at 10 \*\* – 30 \*\*, and just to make PS insolubilize.

[0021]PS insolubilized by addition of metal salt is recoverable by centrifugal separation, filtration, settlement separation, or other means. It is also possible to refine further by the publicly known refining means, for example, column chromatography etc., or other means. Since the content of other phospholipid is falling notably, PS concentrate of this invention can be performed comparatively simple [ such a refining means ].

[0022]PS concentrate of this invention can be prescribed for the patient with the gestalt of drugs, foodstuffs, cosmetics, etc. For example, if it is a case where it uses with the gestalt of the drugs which solicit the physiology effect of phospholipid, a supplement, etc., it can administer orally as liquid preparations, such as solid

preparations, such as a capsule, a granule, a tablet, and powder medicine, or syrups. Even if it is not an orally administered drug, it is also possible to prescribe a medicine for the patient with parenteral gestalten, such as injections, skin external preparations, and a rectum administration agent.

[0023]At the time of manufacture of each pharmaceutical preparation, milk sugar, starch, crystalline cellulose, calcium lactate, Excipients, such as magnesium aluminometasilicate and a silicic acid anhydride, white soft sugar, Binding materials, such as hydroxypropylcellulose and a polyvinyl pyrrolidone, What is necessary is just to use suitably lubricant, such as disintegrator, such as carboxymethyl cellulose and carboxymethyl-cellulose calcium, magnesium stearate, talc, monoglyceride, and a sucrose fatty acid ester, and the other ingredients which may be permitted as medicine and foodstuffs.

[0024]What is necessary is to add what carried out purification treatment of the PS concentrate obtained by the method of this invention as it is or suitably in eating-and-drinking articles, such as fats and oils, a hard candy, fermented milk, a candy, a seasoning, and fish flour, and just to manufacture using a conventional method, in expecting the same physiology effect and using with a common foodstuffs gestalt (gestalt of "clear foodstuffs").

[0025]On the occasion of use with the gestalt of these drugs, foodstuffs, etc., the phospholipid composition in which PS obtained by the method of this invention was condensed can be blended suitably. what is necessary is just to blend suitably the quantity which is a grade which can acquire the effect and problems, such as superfluous ingestion, do not produce, and the quantity the ingestion which is 50 mg - about 1000mg/day is expected to be, if it is a case where the physiology effect of PS is solicited

[0026]As for phospholipid of this invention, it is preferred to use as an emulsifier and to add 0.01 to 10% to drugs, foodstuffs, cosmetics, etc. in that case.

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[Translation done.]

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EXAMPLE

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[Example] Although an example is given to below and this invention is explained to it, this invention is not limited to these.

[0028] while taking 10.0 g of example 1 soybean lecithins (PC80: made by clo KURAN), and the soybean oil 2.0g into a 100-cc medium bottle, adding 90.0 g (100mL) ethyl acetate here and agitating with a stirrer -- warming -- it dissolved. \*\*\*\* picking and 0.1M sodium phosphate buffer solution (pH 7.0) 5.8mL was added, and L-serine 1.2g and PLD-Y1 (Made by Yakult Honsha) 1,500 unit were dissolved.

[0029] The medium bottle containing PC80 solution whole quantity is kept warm at 50 \*\*, the whole quantity of the solution (L-serine+PLD-Y1) which kept it warm at 50 \*\* is added here, and a reaction is started, and it was made to react at 50 \*\* for 5 hours, stirring gently with a stirrer. After ice-cooling for 30 minutes, settling phospholipid and collecting, in boiling water, it was neglected for 20 minutes and the enzyme was deactivated.

[0030] Ethanol 40mL could be added to the collected phospholipid layer, and it mixed, and by neglecting it at 4 \*\* overnight, precipitate was made to form and supernatant liquid was collected. Ethanol 12mL could be further added to the precipitating part, it was neglected for 30 minutes after mixing, supernatant liquid was collected by centrifugal separation, and soybean transition lecithin / ethanol solution was obtained by mixing with previous supernatant liquid.

[0031] Thus, add 0.1M sodium acetate / ethanol solution to the obtained soybean transition lecithin / ethanol solution 2.0mL (about 0.33 g of solid content, PS content in phospholipid = 32.5%) 1.0 mL, and it is neglected at -20 \*\* for 1 hour, Centrifugal operation separated the formed precipitate (60 mg), and it washed by ethanol (PptNa-1). As a result of saving supernatant liquid for several days at -20 \*\*, since the precipitate (7 mg) was produced further, centrifugal operation separated with supernatant liquid (SupNa, hardening-by-drying weight = 227 mg), and it washed by ethanol (PptNa-2).

[0032] After hardening each sample by drying, it dissolves in a diluent solvent (hexane: diethylether : isopropanol =2:2:1), After developing with thin layer chromatography (chloroform: developing solvent : methanol : acetic acid =13:5:2), under [ a fixed quantity / analysis system / gel pattern image / content / phospholipid / it makes phospholipid color with a Dittmer-Lester reagent and ]. A result is shown in the next table 1.

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[0034]

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[0035]Reduced pressure drying of the soybean transition lecithin / the ethanol solution prepared in example 2 Example 1 was carried out, 900 mg of them was taken into the 50-cc medium bottle, and it was made to dissolve in the mixed liquor of chloroform 22.5mL and methanol 15mL. In this way, the prepared soybean transition lecithin / clo meta-solution are poured distributively into every 4.0 mL 6.0-cc medium bottle, 1M saline (a. a lithium chloride, b. potassium chloride, c. sodium chloride, d. magnesium chloride, e. calcium chloride, f. ammonium chloride, g. ammonium sulfate) was added here 0.8 mL. After shaking the bottle and mixing several times, it settled, chloroform layers were collected, and it took in the test tube which carried out weighing of the 1.0mL of them, and dried under nitrogen.

[0036]In this way, to the obtained dry matter (about 50 mg, a phospholipid content ≈ about 25 mg), added 0.10-ml diethylether and dissolved, and added the ethanol of 1.0mL here gradually, precipitation was made to form, the whole suspension was centrifuged, and supernatant liquid and precipitation were divided.

[0037]An ethanol extract applies 5micro of things L by which 5microL and precipitation dissolved the whole for the diluent in chloroform of 2.5mL 2.5 times to a laminated plate, Under [ a fixed quantity / analysis system / gel pattern image / content / phospholipid / it makes phospholipid color after deployment and with a Dittmer-Lester reagent on condition of Example 1 and ]. A result is shown in the next table 2.

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[0043]Diethylether of 5.0mL was added to the obtained dry matter, and it dissolved in it, and the ethanol of 40mL was added here gradually, it dissolved (20 \*\*), the whole suspension was centrifuged, and supernatant liquid (S1) and precipitation (P1) were separated. Diethylether of 5.0mL was again added to P1, and it dissolved in it, and the ethanol of 50mL was added here gradually, it dissolved (20 \*\*), the whole suspension was centrifuged, and supernatant liquid (S2) and precipitation (P2) were divided.

[0044]That to which 5microL and S1 diluted with chloroform what soybean transition lecithin dissolved in 10-mg/mL, and dissolved P2 (damp or wet condition) in chloroform of 20 mg/mL 2.5 times 5microL, And apply undiluted solution 10muL to a laminated plate, phospholipid is made to color after deployment and with a Dittmer-Lester reagent on condition of Example 1, and S2 is the bottom about a fixed quantity of phospholipid contents by a gel pattern image analysis system.

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[0047]Reduced pressure drying of the soybean transition lecithin / the ethanol solution prepared in example 4 Example 1 was carried out, 900 mg of them was taken into the 50-cc medium bottle, and it was made to dissolve in the mixed liquor of chloroform 22.5mL and methanol 15mL. In this way, the prepared soybean transition lecithin / chloroform methanol solution were poured distributively into every 4.0 mL 6.0-cc medium bottle, and 2M salt solution which dissolved in the citrate-sodium-acid-citrate buffer solution (pH 2.8, 3.6, 4.1, 4.6, 5.0) of various pH was added here 0.8 mL. After shaking the bottle and mixing several times, it settled, chloroform layers were collected, and it took in the test tube which carried out weighing of the 2.5mL of them, and dried under nitrogen.

[0048]To the dry matter (about 120 mg), diethylether of 0.2mL was added and it dissolved, and the ethanol of 2.0mL was added here gradually, it dissolved, the whole suspension was centrifuged, and supernatant liquid and precipitation were separated. An ethanol extract that by which 10microL and precipitation dissolved the whole for the diluent in chloroform of 10.0mL 10 times 5microL, Under [ a fixed quantity / analysis system / gel pattern image / content / phospholipid / apply to a laminated plate, and it makes phospholipid color with an after-deployment Dittmer-Lester reagent on condition of Example 1 and ].

[0049]On pH 3.6 or more conditions, both PS and PA were collected by the precipitation fraction ( $PA/PS=0.23$ ), and were not able to separate both. On the other hand, a possibility that it could carry out fractionation of the PS in the state of sodium salt low ( $PA/PS=0.15$ ) relatively if the amount of PA contained in precipitation repeats the ethanol treatment in this condition was shown by pH 2.8.

[0050]The result of having scoured the serine solution 190g (serine 70g+ water 120g)

and the solution (24 mg/mL) of phospholipase D (PLD-Y1, Yakult Honsha Make) to the soybean lecithin 200g of 40% of the example 5PC content 10 mL, and having made them reacting to it at 55 \*\* for 5 hours, The resultant whose PS content in phospholipid is 46.7% was acquired.

[0051]Ethyl alcohol 20mL was added to the resultant 5.0g, and ethyl alcohol 5mL extracted residue (precipitate) twice further after extraction at 45 \*\*. 3 times of extracts are mixed, salt solution 0.20mL is added to 5mL of them 25%, it was neglected to the room temperature and precipitate was made to form after warming at 45 \*\*. As a result, it turned out that it is 62.1% in a sediment and PS is efficiently condensed by the precipitating part to PS content in supernatant liquid having been 3.3% among the dry solid.

[0052]50 mg of sodium acetate powder is added to mixture 5mL of the extract prepared in example 6 Example 5, it was neglected to the room temperature and precipitate was made to form after warming at 45 \*\*. As a result, it turned out that PS is efficiently condensed by the precipitating part as well as the case where are 61.8% in a sediment and a salt solution is used to PS content in supernatant liquid having been 3.5% among the dry solid.

[0053]Added the salt solution 25% to the mixture of an extract prepared in example 7 Example 5, the insoluble matter of PS was made to form, and PS content in precipitate phospholipid and the amount of PS collected by the precipitating part were measured. As the result, the relation between a salt addition, PS recovery rate to precipitate, and PS content in phospholipid is shown in Table 5.

[0054]

[Table 5]

[0055]PS was condensed by the precipitate fraction also on condition of any as shown in Table 5, but the quantity of the salt especially added to per [ phospholipid 1g in an extract mixture ] of PS content in precipitate phospholipid is not less than 55% in the range of 10 or less millimols, and PS was efficiently condensed by the precipitating part. On the other hand, when a salt addition was 0.05 or less millimols, the recovery rate of PS to precipitate is 60% or less, and not less than 40% existed in the supernatant liquid part. Although it was possible to have condensed PS from the above result to precipitate with any addition, the addition of the salt to the phospholipid 1g which dissolved in alcohol was considered to be the addition to which especially the range of 0.15 to 10 millimol was suitable for practical use.

[0056]Reference example It tried to refine PS from the soybean transition lecithin obtained in Example 1 according to the example of refining of PS from the refining bovine brains by CM cellulose column chromatography (the new chemical experiment lecture 4, lipid II phospholipid, p.127).

[0057](1) Preparation of Na<sup>+</sup> type CM cellulose : suction \*\*\*\* was carried out, after adding CM-52 (Whatmann, swollen type) 50g gradually and settling it for about 30 minutes into 0.5N sodium hydroxide 500mL. It washed again after distilled water washed until \*\*\*\* became neutrality (500mLx2 time) until it passed 0.5N chloride 500mL and \*\*\*\* became neutrality with distilled water (500mLx2 time). After settling for 30 minutes in addition, stirring this gel in 0.5N sodium hydroxide 500mL, it washed

until it became neutrality with distilled water (500mLx1 time), and saved as methanol suspension eventually after washing by methanol 500mL further at the room temperature.

[0058](2) A column 30 mm in inside diameter is filled up with CM-52 adjusted by chromatography: (1) (bed volume 70mL). After having dissolved the soybean transition lecithin 1.0g of Example 1 in chloroform of 10mL after 500-mL-pouring chloroform and carrying out a conditioning, and applying the solution except precipitation to a column, you made it eluted under chloroform and the chloroform eluate fraction (Fr.1) of 200mL was obtained.

[0059]Chloroform methanol (85:15, v/v) 400mL (Fr.2+3), Chloroform methanol (75:25, v/v) 400mL (Fr.4+5), Chloroform methanol (65:35, v/v) 400mL (Fr.6+7) and chloroform methanol (50:50, v/v) 400mL (Fr.8+9) were passed, fractionation of every 200 mL was carried out, and silica gel thin layer chromatography analyzed phospholipid.

[0060]As a result, although phospholipid was hardly detected by the chloroform eluate fraction (Fr.1), 0.29g (dry weight) elution of the thing of the almost same presentation as the ethanol precipitation thing applied by the cloudy state was carried out in the first half (Fr.2) of a chloroform methanol (85:15, v/v) fraction. Although PC was not contained in the chloroform methanol (75:25, v/v) fraction (Fr.4+5), both PS and PA are contained, and it was mostly eluted especially in PA. And purity was [ that PA is not contained on and after the second half (Fr.7), but coloring slight at the starting point in addition to PS is only seen, and ] 97.7% although 8.0% of PA was still contained in the first half (Fr.6) of a chloroform methanol (65:35, v/v) fraction. Although a considerable amount of PS was contained in the first half (Fr.8) of a chloroform methanol (50:50, v/v) fraction, there was almost no effluent in the second half (Fr.9).

[0061]Although it became clear from the above result that PS can be refined also from soybean transition lecithin by the cation-exchange chromatography of the almost same conditions as a bovine brain fraction, It turned out that it is the method of not being suitable for a recovery rate obtaining a lot of refining PS very low (it is about 20% when Fr.7 or subsequent ones are collected).

[0062]

[Translation done.]

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